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Abstract: BACKGROUND/AIMS: HNF1B nephropathy is characterized by dominantly inherited renal hypodysplasia with few cysts, slow renal decline and hypomagnesemia. Mice with antenatal inactivation of HNF1B are characterized by polycystic kidneys, renal failure and a profound decrease in cystic gene (Pkhd1, Umod, Pkd2) expression. Mice with inactivation after postnatal day 10 have no renal phenotype. METHODS: Quantification of mRNA expression of HNF1B, six of its potential target genes (PKHD1, PKD1, PKD2, IFT88, TMEM27 and UMOD) and three genes involved in the Mg(2+) renal homeostasis (ATP1A1, FXYD2 and CLDN16) in the urinary sediment of 11 individuals with mutation of HNF1B and in 9 controls (non-invasive assessment of the renal transcriptome). RESULTS: As compared to controls, no difference was observed in the urinary mRNA amount of HNF1B and the renal cystic genes. A significant increase in the expression of ATP1A1, which encodes the 1-subunit of the Na(+)/K(+)-ATPase, was identified in HNF1B patients consistent with its role in Mg(2+) homeostasis. CONCLUSION: Assessment of mRNA expression in urinary sediment is a non-invasive method applicable to gain insights into the pathophysiology of inherited nephropathies in humans. HNF1B nephropathy is generally not associated with postnatal down-expression of renal cystic genes in human, a finding consistent with mouse models.

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Expression of Renal Cystic Genes in Patients with *HNF1B* Mutations

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Key Words
HNF1B · Urinary mRNA · Inherited renal disease · Hypomagnesemia · Tubulopathy

Abstract
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Introduction

In humans, *HNF1B* (hepatocyte nuclear factor-1β) mutations result in a multisystem disease with autosomal dominant inheritance. In accordance with the role of *HNF1B* during development, the pancreas, genital tract, liver and kidneys may all be affected, resulting in maturity-onset diabetes of the young type 5, genital malformations, liver tests abnormalities and nephropathy [1–3]. In mouse kidney, *Hnf1b* is expressed in all the nephron segments, from proximal tubule to collecting duct [4]. Renal phenotype in *HNF1B*-related disease is highly heterogeneous. It mostly encompasses (1) bilateral renal hypodysplasia most often translating into hyperechogenic kidneys or renal cortical cysts from the an-

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tenatal period through childhood; (2) in adulthood, renal cysts are frequently lacking or few, and (3) at any age, the nephropathy harbors a profile of chronic tubulointerstitial nephritis with slowly progressive renal decline, low-range proteinuria without hematuria, and a distinctive renal loss of magnesium and potassium [1, 5, 6]. In addition, we and others have emphasized a wide inter- and intra-family variability [5, 7]. Beside whole-gene deletions, which account for more than half of all mutations [8], private heterozygous point mutations of *HNF1B* have been reported, including missense, nonsense, frameshift and splicing mutations [7]. Molecular mechanisms underlying the clinical heterogeneity are currently unknown and may involve stochastic effects or modifying genes.

While *Hnf1b*$^{+/-}$ mice are fertile and do not exhibit renal involvement, *Hnf1b*$^{-/-}$ die early in embryogenesis due to the lack of visceral endoderm differentiation [9]. In mice, biallelic renal-specific *Hnf1b* invalidation leads to polycystic kidney disease and early renal failure [10]. Gresh et al. [10] showed that *Hnf1b* controls a large transcriptional network including genes coding for proteins that co-localize to the primary cilium, a structure involved in epithelial cells proliferation and planar cell polarity [11]. Hence, a significant down-regulation of the renal expression of *Tmem27*, *Ift88*, *Umod*, *Pkd2* and *Pkhd1* was observed in this model [10, 12]. Given their implication in renal cystic diseases, these genes were collectively referred to as 'renal cystic genes'. More recently, Verdeguer et al. [13] showed that renal-specific invalidation of *Hnf1b* after postnatal day 10 was not followed by a down-regulation of these renal cystic genes, except for the *Umod* gene, while transient increase in epithelial cell proliferation (induced by ischemic acute kidney injury) promoted cystic kidney disease in this model.

In an attempt to confirm these findings in humans and to decipher the molecular mechanisms of magnesium renal loss, we used a direct approach relying on mRNA expression analysis in urinary sediment. This method has been established as a non-invasive tool to diagnose renal allograft rejection [14, 15] and more recently, to determine gene expression in podocytes and tubular epithelial cells in the mouse [16, 17] and in humans [3, 18]. We assessed the expression of *HNF1B* and of six cystic genes *PKHD1*, *PKD1*, *PKD2*, *IFT88*, *TMEM27* and *UMOD* in urinary cells of 11 *HNF1B*-mutation carriers (*HNF1B* patients) and 9 controls. We extended the analysis to urinary expression of *ATP1A1*, *FXYD2* and *CLDN16*, which respectively encode for the α1 and the γ-subunit of the Na^+/K^+-ATPase and claudin-16. These genes are involved in magnesium homeostasis and the latter two have been found mutated in familial hypomagnesemia [19, 20]. Limited availability of mRNA did not allow to assess the mRNA of *TRPM6*, *EGFR* and *KV1.1*, three additional genes involved in the fine tuning of Mg^{2+} in the distal convoluted tubule [21–23].

Patients and Methods

Patients and Controls

Patients were recruited in the Renal Pediatric Unit at University Hospital of Toulouse, France. Genotyping of *HNF1B* was performed in all patients as previously recommended [8]: quantitative multiplex PCR of short fragment (QMPSF) was applied to identify gene deletion, followed by direct sequencing of the nine exons and all exon-intron boundaries in patients with normal QMPSF.

Clinical history of renal and extrarenal involvement in the *HNF1B* patients was recorded through a standardized assessment of the patient's hospital records. According to age, estimated glomerular filtration rate (eGFR) was calculated using the simplified MDRD formula or Schwarz formula in individuals <16 years old. Hypomagnesemia was defined by serum magnesium level <0.75 mmol/l. Imaging studies of the kidney consisting of ultrasonography and/or computed tomography were recorded. Diabetes was diagnosed on the basis of receiving either insulin or oral agents, or biochemical evidence of diabetes in accordance with WHO guidelines. Pancreas imaging (ultrasonography before 15 years of age and computed tomography scan thereafter) was performed in all patients.

Controls consisted of 9 healthy young individuals [5 males and 4 females, median age 38 years (25–47)]. Seven were first-degree relatives of the *HNF1B* cohort, for whom genetic testing ruled out a mutation of *HNF1B*. According to the French law, informed written consent was obtained from all subjects and tested relatives, and the study was conducted in agreement with the principles of the Declaration of Helsinki.

Immunostaining of Urinary Cells

Freshly voided urine (100 ml) of a control individual was centrifuged at 700 *g* for 10 min. Supernatant was carefully aspirated and the sediment pellets was washed twice with phosphate-buffered saline (PBS). The pellet was then resuspended in 500 μl of PBS. The resuspended sediment was then spun onto slides, which were air-dried and then fixed with 3% paraformaldehyde at room temperature for 15 min. After washing with PBS, the slides were incubated with anti-human cadherin-16 antibody (Abcam ab64868) at a dilution of 1:100 for 60 min. After being washed with PBS, a biotin-conjugated secondary antibody and subsequent streptavidin-biotin complex formation was performed according to manufacturer's instructions. The sediment was counterstained with DAPI nuclear stain to distinguish whole cells from cells fragments.

Urinary Cell RNA Isolation

To avoid technical bias related to urinary sampling, 2–5 (median 3) diurnal micturitions [median volume 80 ml (25–150)]

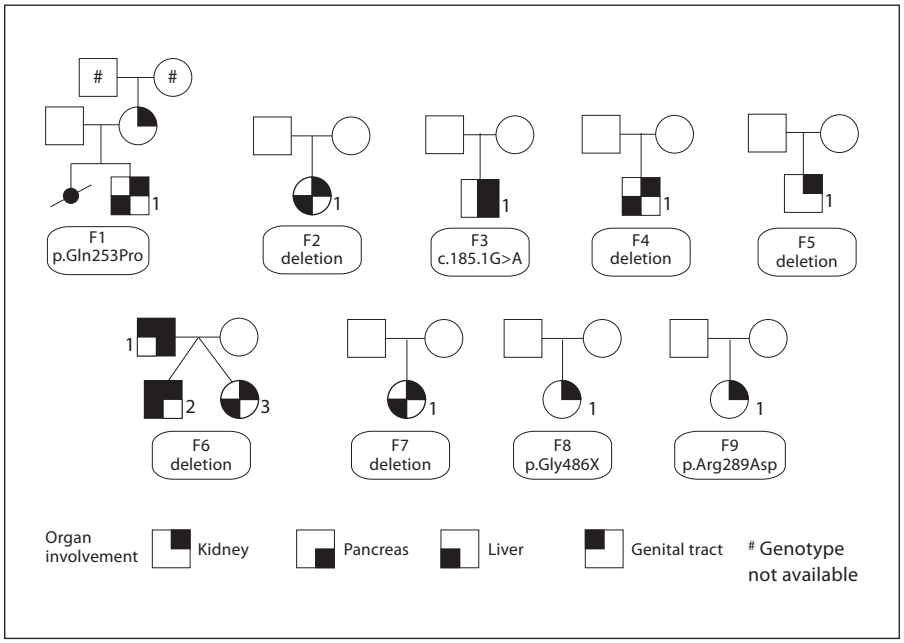

Fig. 1. Summary of phenotype and genotype changes in nine French families with *HNF1B* mutation. Individuals recruited for urinary transcriptome analysis in the study are identified by a number.

were collected from each patient. Isolation and lysis of urinary cells and RNA extractions were performed using the *ZR Urine RNA isolation kit*™ (Zymo Research, Orange, Calif., USA) according to the manufacturer's protocol. RNA quality was assessed by measurement of the optical density 260/280 ratio with a target optical density ratio >1.7, as previously recommended [14].

Real-Time Quantitative PCR

cDNA were generated from urinary cell RNAs and quantified using the Verso™ *SYBR Green 2-Step qRT-PCR Fluorescein* reagent (ThermoScientific-Fisher, Illkirch, France). The primers were designed using Primer3 software [http://biotools.umassmed.edu/bioapps/primer3_www.cgi]. Their sequence is provided in online supplementary table 1 (for all suppl. material, see www.karger.com/doi/10.1159/000334954). The efficiency of each set of primers was assessed by dilution curves and the Ct differences between the reference *(GAPDH)* and target genes calculated for each sample of each patient. The formula used to quantify the relative changes in target over *GAPDH* mRNAs between the two groups is derived from the $2^{-\Delta\Delta Ct}$ formula recommended by Pfaffl [24]. The RT-PCR conditions were as follows: 51°C for 60 min, 95°C for 2 min. q-PCR was carried out on an ABI PRISM® 7600 sequence detection system using SYBR® green with the following conditions: 95°C for 15 min followed by 40 cycles (95°C for 30 s, 58°C for 30 s, and 72°C for 1 min).

Statistical Analysis

Data are presented as absolute medians and limits. Significant differences between *HNF1B* patients and control were tested by the Mann-Whitney test. p values <0.05 were considered significant.

Results

Clinical, Genetic and Biological Characteristics of HNF1B Patients

We studied 11 *HNF1B* patients (7 children and 4 individuals ≥16 years old; 7 males and 4 females) at a median age of 4 years (range 2–40), belonging to nine unrelated families (fig. 1). Familial screening indicated that the mutation occurred de novo in seven families. Molecular analysis of *HNF1B* identified a whole-gene deletion in 7 patients and point mutations in 4 (c.185-1A>G, p.Gln253Pro, p.Asn289Asp and p.Gly456X), a distribution observed in previous studies [3, 8].

Phenotypes of the *HNF1B* patients are summarized in table 1. Renal disorder was the first symptom identified in all patients except in patient 6.1 who presented with infertility and genital tract malformation. Briefly, 4 out of the 11 patients had chronic renal failure (CKD stage 1–3) according to their estimated creatinine clearance (23, 38, 45 and 64 ml/min at 16, 18, 21 and 40 years of age, respectively). Low serum magnesium (0.61–0.70 mmol/l) was found in 4 patients. All of them had increased urinary magnesium excretion (as defined by the ratio of magnesium to creatinine clearances) from 5 to 9%.

Renal imaging detected a solitary kidney in 6 individuals, including 2 with hypoplastic kidney (patients F1.1 and F2.1); in 4, yearly abdominal imaging demonstrated slow involution of their multicystic dysplastic kidney. Among

Table 1. Clinical and genetic characteristics of 11 individuals from nine unrelated families with *HNF1B* mutation

Patient	Age years	Gender	Mutation	Kidney malformations	CRF	Serum Mg^{2+} level (N: 0.75–1 mM)	Pancreas abnormalities	Liver test abnormalities	Genital tract abnormalities
F1.1	3	M	p.Glu253Pro	Hypoplastic horseshoe kidney, few cysts, HE	No	Normal	No	Yes	NA
F2.1	4	F	Deletion	Right: involution Left: hypoplastic, one cyst, HE	No	Normal	No	Yes	NA
F3.1	16	M	c.185–1G>A	Right: normal Left: involution	Yes	0.7 mmol/l	Hypoplastic Low fecal elastase	No	NA
F4.1	4	M	Deletion	Right: MCDK, HE Left: hypoplastic	No	0.61 mmol/l	No	Yes	NA
F5.1	18	M	Deletion	Right: hypoplastic, HE Left: hypoplastic, HE	Yes	Normal	No	No	NA
F6.1	40	M	Deletion	Right: few cysts, pyelic dilatation Left: absent	Yes	0.65 mmol/l	Hypoplastic	No	BAVD Ectopic testis
F6.2	4	F	Deletion	Right: few cysts, HE Left: few cysts, HE	No	Normal	No	Yes	Ectopic testis
F6.3	4	M	Deletion	Right: few cysts, HE Left: few cysts, HE	No	0.7 mmol/l	No	Yes	NA
F7	4	M	Deletion	Right: few cysts, HE Left: few cysts, HE	No	Normal	No	Yes	NA
F8	2	F	p.Gly456X	Right: involution Left: few cysts, HE	No	Normal	No	No	NA
F9	21	F	p.Arg289Asp	Right: absent Left: normal	Yes	Normal	No	No	No

CRF = Chronic renal failure (eGFR <60 ml/min/1.73 m^2, according to simplified MDRD formula, or Schwarz formula in individuals <16 years); HE = hyperechogenicity; MCDK = multicystic dysplastic kidney; BAVD = bilateral absence of vas deferens; NA = not available.

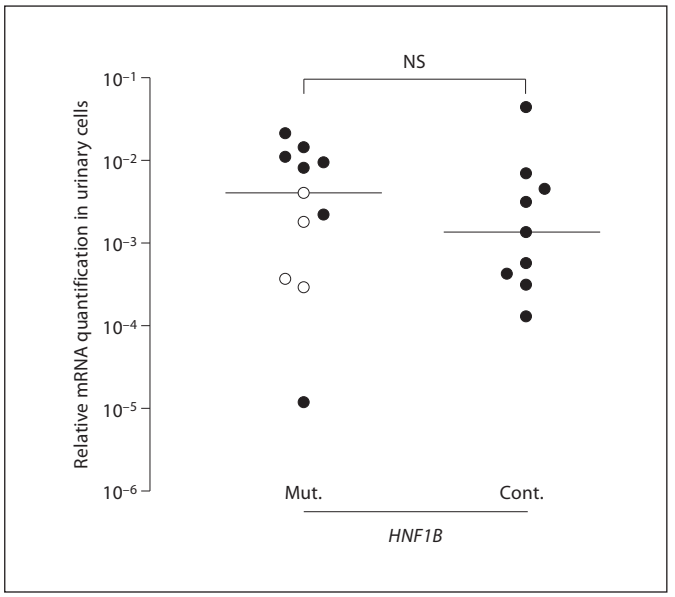

Fig. 2. mRNA levels of *HNF1B* in urinary cells of 11 patients with *HNF1B* mutation and 9 control individuals. Dots represent the quantification levels of *HNF1B* normalized mRNA ($2^{-\Delta\Delta Ct}$) relative to the quantity of *GAPDH* transcripts. ● = Whole-gene deletion; ○ = point mutation.

the remaining patients, imaging showed unilateral hypoplastic kidney (n = 1), bilateral hypoplastic kidneys (n = 1) or bilateral normal sized kidneys (n = 3). Renal cysts were observed in 8 individuals (multicystic dysplastic kidney in 1 and few cysts in 7). Renal hyperechogenicity was identified in 8 individuals (mainly of pediatric age). Patient 6.1 had a solitary ectopic kidney with few cysts diagnosed at age 36, while his 2 children had hyperechogenic kidneys with few cysts diagnosed antenatally.

mRNA Expression in Urinary Sediment

Using immunostaining of urinary cells with an antibody directed against the kidney-specific cadherin, cadherin-16, we first confirmed in a control individual that renal tubular cells may be collected and analyzed from urine sample (see supplementary data). The renal/urothelial cell ratio was almost 1/10. After urinary cell isolation and lysis, mRNA was extracted and reverse transcribed. Thereafter, we confirmed that renal cells collected in urine come from the different tubular segments, as demonstrated by RT-PCR using specific primers of *AQP1*, *UMOD*, *CLDN16*, *AQP2* and *AVPR2* (data not shown). Leukocyturia was ruled out in all patients with urinary dipstick.

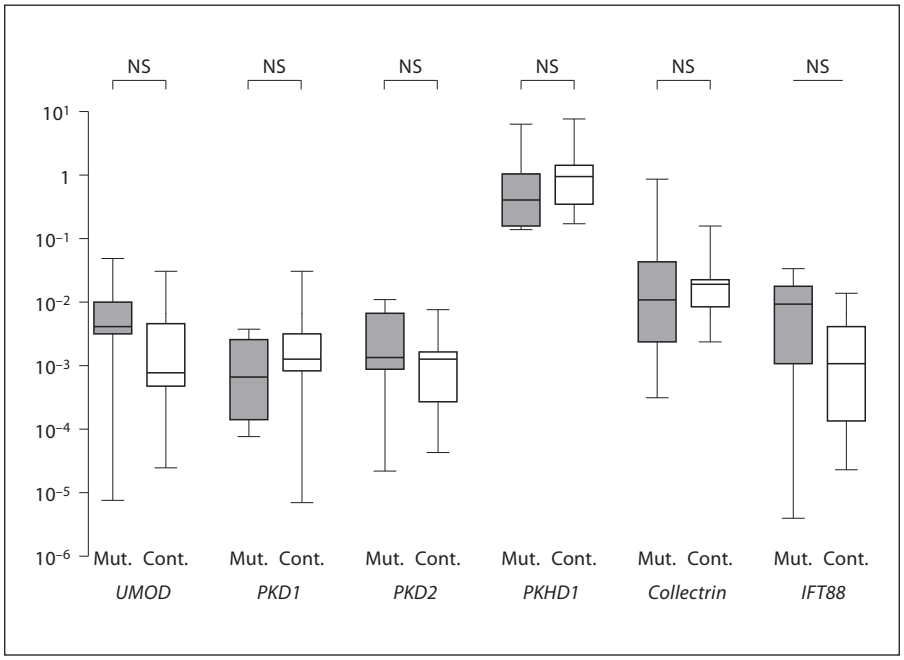

Fig. 3. mRNA levels of *UMOD, PKD1, PKD2, PKHD1, TMEM27* and *IFT88* in urinary cells of 11 patients carrying a mutation of *HNF1B* and 9 control individuals. The box-plot representation shows the medians and the percentile values 10, 25, 75, and 90 for the quantification levels of normalized mRNA ($2^{-\Delta\Delta Ct}$) relative to the quantity of *GAPDH* transcripts.

Relative quantification of the expression of *HNF1B* mRNA in urinary cells showed no difference in transcript levels between *HNF1B* patients and controls (fig. 2). In the subgroup of 7 patients with *HNF1B* whole-gene deletions in whom a decrease of expression levels of 50% could be anticipated from haploinsufficiency, no significant difference in *HNF1B* expression was detected.

We next examined the expression of potential target genes of *HNF1B* in humans and observed that the expression of *UMOD*, *PKHD1*, *PKD1*, *PKD2*, *TMEM27* and *IFT88* mRNAs were not statistically different between patients and controls (fig. 3). Neither the age at enrolment into the study nor the type of *HNF1B* mutation affected relative expressions of these genes (data not shown).

Finally, we assessed the expression of three additional transcripts involved in magnesium homeostasis, *ATP1A1* (encoding for the α1-subunit of the Na^+/K^+-ATPase), *FXYD2* (encoding for the γ-subunit of the Na^+/K^+-ATPase) and *CLDN16* (which encodes for claudin-16). The normalized expression of *ATP1A1* mRNA was significantly higher in *HNF1B* patients compared to controls (sixfold, $p = 0.009$) while no difference was detected in expression of *FXYD2* and *CLDN16* (fig. 4). *ATP1A1, FXYD2* and *CLDN16* mRNA expression was not correlated with serum magnesium level (data not shown).

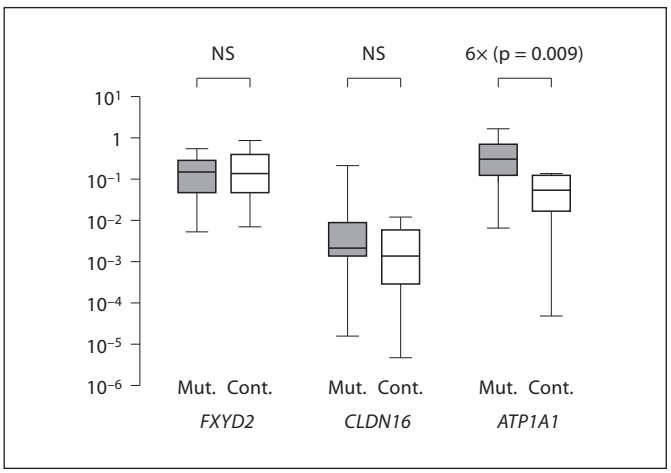

Fig. 4. mRNA levels of *FXYD2, CLDN16* and *ATP1A1* in urinary cells of 11 patients carrying a mutation of *HNF1B* and 9 control individuals. The box-plot representation shows the medians and the percentile values 10, 25, 75, and 90 for the quantification levels of normalized mRNA ($2^{-\Delta\Delta Ct}$) relative to the quantity of *GAPDH* transcripts.

Discussion

To investigate whether *HNF1B*-related nephropathy is associated with a peculiar postnatal profile of renal cystic genes in human, we performed a non-invasive expression

analysis following collection of urinary cells in 11 patients aged between 2 and 40 years. We also used this method to investigate whether hypomagnesemia observed in patients with *HNF1B*-related nephropathy is associated with differences in expression of genes involved in Mg^{2+} metabolism. The main limit of this study is the high degree of variability of the tubular to urothelial cells ratio that may be collected in urine samples. Subsequently, high variability of expression levels was observed when GAPDH was used as reference gene. Because renal transcriptomic changes are currently unknown in patients with *HNF1B* mutations and *Hnf1b* may modify expression of segment-specific tubular markers in the mouse, we could not use them as reference gene. Note, previous studies have shown that GAPDH may be used as reference gene to identify intra-renal mRNA changes [17]. Lastly, we did not use *HNF1B* itself as reference gene because of its expression in urothelial cells [4] and the lack of data concerning its expression in each tubular segment in adult normal and *HNF1B* patients.

In these 11 patients with heterogeneous renal phenotype, including 7 with a whole-gene deletion, we did not observe any change in expression of *HNF1B* mRNA as compared to controls, suggesting that after antenatal period, one copy of *HNF1B* could be sufficient to reach normal expression of this gene in postnatal kidney.

The lack of down-regulation of renal cystic genes may account for the low cystic involvement in most patients of this series diagnosed with *HNF1B*-related nephropathy in adulthood, as exemplified in patients F3.1, F5.1, F6.1 and F9, in whom imaging studies exhibited no or few renal cysts. This is reminiscent of pathological findings from our early experience: rare cysts of glomerular or tubular origin were observed on kidney specimens from 6 unrelated individuals [1].

The lack of postnatal detectable difference in expression level of *HNF1B* and related cystic genes in *HNF1B*-mutated individuals is not entirely unexpected according to previous findings. First, genetic discrepancies between mouse models and human disease suggest that the underlying molecular mechanisms are different. In contrast to rodent models, which share recessive inheritance at tissue level [9, 10, 12], *HNF1B*-related human disease is dominantly inherited [25]. While a significant down-regulation of the expression of renal cystic genes *Tmem27*, *Ift88*, *Umod*, *Pkd2* and *Pkhd1* was observed postnatally in mice with antenatal renal-specific conditional inactivation of *Hnf1B* [10, 12], a sustained expression of *PKD1*, *PKD2* and *PKHD1* was observed in epithelial cells layering both cystic and non-cystic renal structures of two *HNF1B*-mutated human fetuses [26]. This could be related to the persistent expression of the non-mutated allele. Moreover, there is no evidence for a two-hit process within renal tissue in *HNF1B* individuals, with the exception of somatic deletion of the second allele in neoplastic tissue of the rare individuals who developed chromophobe renal carcinoma superimposed on *HNF1B* nephropathy [27]. Second, target genes of transcription factors may widely diverge between humans and mice. This applies to the HNF family, with recent recognition of a 40–80% divergence among HNF1A and HNF4A target genes between mouse and human [28]. Of note, the overexpression of *HNF1B* in a human embryonic kidney cell line (HEK293) was not followed by an up-regulation of the target genes of *Hnf1b* previously identified in a mouse model [29]. Finally, rodent models have recently shed light on the key role of *Hnf1b* in the transcriptional switch occurring at the end of the kidney maturation process, around postnatal day 10 (P10) [13]. While conditional renal-specific inactivation of *Hnf1b* in embryos and neonates until P3 results in severe polycystic kidney disease, no cystic lesions develop in mice induced at P10 or later and renal transcriptome analysis demonstrated a sustained expression of cystic genes *(Pkd2*, *Pkhd1*, *Kif12*, *Tmem27)*. This finding is consistent with the lack of difference of urinary mRNA amounts of *PKHD1*, *PKD2* and *TMEM27* between patients and controls investigated in this study. Our study highly suggests that the expression of most cystic genes in human quiescent renal cells is not dependent of *HNF1B*. Thus the exact role of *HNF1B* in adult human kidney remains to be defined.

In adults, mutations of *HNF1B* may be associated with low serum magnesium or low serum potassium level due to renal loss of either ion [5, 30]. These peculiar findings have not been reported so far in animal models, which hampers molecular analysis. Among the 11 patients tested in this series, urinary loss of magnesium and potassium were identified in 4 and 2 individuals, respectively. Renal magnesium homeostasis is a complex physiological process that involves many transporters and receptors (e.g. claudin-16, claudin-19, Na^+/K^+-ATPase) all along the different tubular segments (see review by Naderi and Reilly [31]). We tested here the expression of *CLDN16* (claudin-16), *FXYD2* and *ATP1A1* (encoding for the γ-subunit and the α_1-subunit of the Na^+/K^+-ATPase) in renal tubular cells of *HNF1B* patients. We found a sixfold increase in mRNA of *ATP1A1* of *HNF1B* patients while mRNA expression of *CLDN16* and *FXYD2* were not different in patients and controls (fig. 4). At first glance, the up-regulation of the Na^+/K^+-ATPase seems to be consis-

tent with its role for maintenance of the transepithelial electrical gradient required for renal magnesium paracellular reabsorption. However, whether *ATP1A1* is a direct target of HNF1B in the human kidney remains unknown. Using the Consite program [http://asp.ii.uib.no:8090/cgi-bin/CONSITE/consite], we failed to identify any putative *HNF1B* binding site in the 4,000 bp preceding the first *ATP1A1* exon. Changes in the level of *ATP1A1* expression may be a functional consequence of hypomagnesemia rather than a direct effect of *HNF1B*, since mutations of *ATP1A1* have not yet been described in humans. Recently, in our study performed before a report showing that *HNF1B* increased expression of a specific isoform of *FXYD2* in vitro [30], we failed to show a decrease in urinary amounts of *FXYD2* mRNA in *HNF1B*-mutated patients but we only assessed the overall expression of *FXYD2* in urinary cells regardless of various isoform-specific expressions. Limited availability of mRNA did not allow us to assess the relative expression of the various isoforms of *FXYD2*.

In summary, we show here that measurement of mRNA expression in urinary sediment is a valuable approach to assess the renal epithelial transcriptome in inherited renal diseases. We demonstrate that the expression of cystic renal disease genes, previously recognized as target genes of *Hnf1b* in mouse, are not dramatically reduced in postnatal (quiescent) renal epithelial cells of human patients carrying a mutation of *HNF1B*, thus confirming distinct functions of the *HNF1B* transcription factor during nephrogenesis and in the mature kidney. Further studies will have to confirm and expand these preliminary results obtained in a small cohort of patients in order to decipher the transcriptional network involving *HNF1B* in postnatal human kidney.

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